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Lymphocyte telomere length is long in *BRCA1* and *BRCA2* mutation carriers regardless of cancer-affected status

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Abstract

Background—Telomere length has been linked to risk of common diseases, including cancer, and has previously been proposed as a biomarker for cancer risk. Germline *BRCA1* and *BRCA2* mutations predispose to breast, ovarian and other cancer types.

Methods—We investigated telomere length in *BRCA* mutation carriers and their non-carrier relatives and further examined whether telomere length is a modifier of cancer risk in mutation carriers. We measured mean telomere length in DNA extracted from whole blood using high-throughput Q-PCR. Participants were from the EMBRACE study in the UK and Eire (n=4,822) and comprised *BRCA1* (n=1,628) and *BRCA2* (n=1,506) mutation carriers and their non-carrier relatives (n=1,688).

Results—We find no significant evidence that mean telomere length is associated with breast or ovarian cancer risk in *BRCA* mutation carriers. However, we find mutation carriers to have longer mean telomere length than their non-carrier relatives (all carriers vs. non-carriers, *P*-trend=0.0018), particularly in families with *BRCA2* mutations (*BRCA2* mutation carriers vs. all non-carriers, *P*-trend=0.0016). Our findings lend little support to the hypothesis that short mean telomere length predisposes to cancer. Conversely, our main and unexpected finding is that *BRCA* mutation carriers (regardless of cancer status) have longer telomeres than their non-mutation carrier, non-cancer-affected relatives. The longer telomere length in *BRCA2* mutation carriers is consistent with its role in DNA damage response.

Conclusions—Overall, it appears that increased telomere length may be a consequence of these mutations, but is not itself directly related to the increased cancer risk in carriers.

Impact—The finding that mutation carriers to have longer mean telomere lengths than their non-carrier relatives is unexpected but biologically plausible and could open up new lines of research into the functions of the BRCA proteins. To our knowledge, this is the largest study of telomere length in *BRCA* mutation carriers and their relatives. The null cancer-risk association supports recent large prospective studies of breast and ovarian cancer and indicates that mean telomere length would not be a useful biomarker in these cancers.

Introduction

Human chromosomes are capped and stabilised by telomeres, comprising several thousand $(TTAGGG)_n$ repeats and a plethora of structural proteins (1-3). Telomere length shortens with each cell division, leading to a progressive decrease with age (4-7) and rare mutations in telomere maintenance genes, such as *TERT*, can cause dramatically-shortened telomeres and premature aging (8,9). It has therefore been hypothesised that short mean telomere length may predispose to a number of diseases of aging, including cardiovascular disease (10-13) and cancer, and thus could be used as a biomarker of disease risk (14). The association of cancer risk with mean telomere length, measured in DNA from leukocytes, has been evaluated in a number of studies, but the results have been inconclusive. Retrospectively-collected studies, where blood samples for telomere length analysis have been taken after cancer diagnosis, have generally found cancer patients to have shorter telomeres than unaffected controls (15-18). However, results from more appropriate prospective study designs, with blood collected prior to diagnosis, have been largely null (16,19-21). In fact, the largest prospective study yet published, of 3,142 cancers from a general population study of 47,102 Danish individuals, reported a correlation between shorter telomere length and a very modest yet significant *decrease* in breast cancer risk (22).

Mutations in *BRCA1* and *BRCA2* confer high risks of breast, ovarian and other cancers. *BRCA1* and *BRCA2* are integral to the early stages of DNA damage recognition and repair (23); BRCA1 is activated by ATR and is involved in cell cycle arrest and replication fork stalling (with CHEK2), and breakage site stabilization (with BRIP1 and BARD1) through directly binding the damaged DNA (24,25). BRCA2 is activated by ATM and recruited to the repair site indirectly via BRCA1, where it stimulates the recruitment of RAD51, a protein integral to repair through homologous recombination and Holliday junction formation (26).

To date, few other studies have examined telomere length in *BRCA1* and *BRCA2* mutation carriers. Martinez-Delgado *et al*. (27). reported shorter telomere length in cancer in *BRCA1* and *BRCA2* carriers compared with sporadic breast cancer, and an earlier age of cancer onset, and shorter age-adjusted telomere length, in successive generations of cancer patients. The same group recently reported retrospectively-collected sporadic (n=178) and hereditary (n=168) ovarian cancer cases to have shorter telomeres when compared with 267 control samples (28).

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In this study, we have evaluated the hypothesis that short telomere length predisposes to breast or ovarian cancer by examining mean telomere length in *BRCA1* and *BRCA2* mutation carriers from the EMBRACE study in the UK and Eire. We have compared mean telomere length between mutation carriers who have been diagnosed with breast or ovarian cancer, and as yet unaffected carriers (who remain at high risk of developing cancer in the future). To further evaluate the hypothesis that mutation carriers (affected or unaffected) might display shortened telomeres, we have compared mean telomere length between *BRCA1* and *BRCA2* mutation carriers and unaffected, mutation-free members from the same families.

Materials and Methods

Study populations

Mean telomere length was determined in blood DNA from participants in the EMBRACE study, an epidemiological study of *BRCA1* and *BRCA2* mutation carriers and their relatives (29). The study began recruiting in 1996 through clinical genetics centres in the UK and Eire. Eligible participants were either confirmed mutation carriers, had been (or were in the process of being) tested for *BRCA* mutations (in families where a pathogenic mutation had been found) and had been found to be a non-carrier, or had attended genetic counselling, had been offered testing, but had declined. The present analysis is based on only proven mutation carriers and non-carrier relatives from EMBRACE.

All participants were over 18 years old and were asked at baseline recruitment to provide a blood sample for DNA analysis, and to complete a comprehensive lifestyle and general health questionnaire. These data were collected to identify any genetic or environmental factors, or surgical interventions, that may modify cancer risks for *BRCA1* and *BRCA2* mutation carriers and their relatives.

In total, mean telomere length data were available for 4,822 subjects; 3,134 mutation carriers (1,628 with *BRCA1* mutations and 1,506 *BRCA2* mutations) and 1,688 non-carrier relatives. Of these 3,134 mutation carriers, 439 were male and 2,695 were female. Of the female carriers, 1,494 were known to have been diagnosed with breast or ovarian cancer and 1,201 were unaffected. Further details are given in Table 1. Twelve percent of the total cancer cases studied presented with ovarian cancer, with the majority 88% having breast cancer as the primary diagnosis, so the two cancer types were pooled for analysis. Cancer diagnoses were predominantly at baseline recruitment (94% of breast cancer cases and 85% of ovarian cancer cases) rather than by follow-up or flagging, but all cases were eligible for our analysis, regardless of the timing of presentation as we had much less power to detect effects in the follow-up and flagging groups separate from baseline. Ethical approval was obtained and all participants gave informed consent.

Telomere length measurement

Relative mean telomere length was ascertained by a SYBR® Green real-time PCR using a version of the published Q-PCR protocols (15,30) modified as described previously (26). In brief, genomic DNA was extracted from whole blood and telomere length was ascertained

through the ratio of detected fluorescence from the amplification of telomere repeat units (TEL) relative to that of a single-copy reference sequence from the β-Globin gene (CON). Telomere and control reactions were performed separately. For each assay, the PCR cycle at which each reaction crossed a predefined fluorescence threshold was determined (Ct value). The difference in the Ct values, ΔCt = Ct TEL – Ct CON, was the measure of telomere length used in the analysis. We were not able to generate absolute telomere length values using these data as calibration samples of known length were not available.

Sixteen percent of the study was run in duplicate, with repeated samples assayed in a secondary run during the experiment, using a separately-prepared mix of PCR reagents. Failed PCR reactions were not repeated. A standard plate of 'test' samples was additionally assayed with each study. This plate consisted of 94 high yield DNA samples and was assayed in each PCR batch, performed as a method of inter-experiment quality control.

The correlation between repeated ΔCt measurements of the same study subjects, assayed in separate PCR batches, was 0.87. The Spearman rank order correlation of the triplicate 'test' plate ΔCt measurements was 0.71. Greater than 93% of the samples attempted gave useable mean telomere length measurements. In unaffected subjects, ΔCt increased with age with an estimated increase 'per annum' ($\bar{o}\Delta Ct$) = 0.0033 (95%CI 0.0015 - 0.0051); *P*-trend=2.8×10^{-4}, after adjustment for carrier status, study plate, relatedness and gender. This is consistent with the established reduction in mean telomere length with age, and the magnitude of the change is consistent with that observed in previous studies

Statistical methods

The intra-experimental quality control comparisons of duplicated samples were assessed using the Pearson product-moment correlation coefficient. The inter-experimental comparison of standard 'test' plates, for assurance of batch-to-batch quality control, was assessed using Spearman's rank correlation coefficient. Prior to all analyses, 'outlier' samples were removed if the CON PCR Ct value was more than two standard deviations from the mean, and these reactions were considered 'fails'.

The association of ΔCt with age at blood draw was evaluated in cancer-free individuals using linear regression, adjusting for age, study plate, gender, and clustered by relatedness. Similarly, the association between mutation carrier status and mean telomere length (ΔCt) was analysed using linear regression, showing the difference in mean telomere length (δΔCt) comparing mutation carriers with non-carriers, with associated 95% confidence intervals (95%CI). The analysis was adjusted for age, study plate, gender, and clustered by relatedness.

The association between disease status in female mutation carriers and telomere length was assessed using a weighted cohort analysis (31-33) Individuals were censored at the age of the first breast cancer diagnosis, ovarian cancer diagnosis, bilateral prophylactic mastectomy or the age at last observation. Weighted Cox regression was used to adjust for the non-random sampling of the mutation carriers with respect to disease status.[31] For this purpose, affected and unaffected individuals were allocated differential weights according to breast or ovarian cancer status, such that the weighted cohort mimics a 'true' cohort of mutation

carriers (32,33). These weights were generated for this study based on time at risk before age at censoring, affected status (breast or ovarian) and mutation type (*BRCA1* or *BRCA2*). This approach has been shown to provide unbiased estimates of the relative risks, adjusting for the oversampling of affected individuals, while utilising the whole dataset. Subjects were categorized into quartiles for telomere length, the boundaries of which were defined by the continuous distribution of ΔCt in the unaffected mutation carrier sample population; the Q1 reference quartile group had the longest mean telomere length and the Q4 quartile group had the shortest. The analysis was additionally adjusted for study plate and age at blood draw, and clustered by family to allow for the non-independence between family members. Male mutation carriers (n=439), carriers of unknown cancer status, and individuals on whom appropriate censoring data were not available were excluded from these analyses

All analyses were performed using Intercooled Stata 11.2 statistical package (Stata, College Station, TX).

Results

The association of mean telomere length with cancer status in *BRCA1* and *BRCA2* mutation carriers

The differences in telomere length between mutation carriers diagnosed with breast or ovarian cancer and unaffected mutation carriers are shown in Table 2. In a weighted Cox regression analysis, no significant associations were detected between telomere length quartiles and the risk of developing either breast or ovarian cancer in *BRCA1* or *BRCA2* mutation carriers (Table 2). In addition, no significant trends were observed by quartile of mean telomere length (*P*-trend=0.76 for *BRCA1*, *P*-trend=0.27 for *BRCA2*.

Comparison of mean telomere length in *BRCA1* & *BRCA2* mutation carriers and their non-carrier relatives

The estimated differences in telomere length between *BRCA1* and *BRCA2* mutation carriers and non-carriers, adjusted for age, study plate, relatedness and gender, are shown in Tables 3 and 4. Heterozygous carriers of *BRCA1* and *BRCA2* mutations had longer telomeres than non-carriers (as shown by a negative covariate-adjusted β coefficient); δΔCt = −0.056 (95%CI −0.091 to −0.021), *P*=0.0018; Table 3. This association was more significant for *BRCA2* mutation carriers (vs. all non-carriers; δΔCt = −0.067 (95%CI −0.108 to −0.026), *P*=0.0016) compared with those with *BRCA1* mutations (vs. all non-carriers; δΔCt = −0.038 (95%CI −0.079 to −0.003), *P*=0.068). The effect sizes for associations between telomere length and mutation status remained virtually unchanged when the analysis was restricted to *BRCA1* and *BRCA2* mutation carriers who had not developed breast or ovarian cancer (but remained at high risk of doing so) and cancer-free, non-carrier relatives (Table 4; *P*-trend=0.011).

Discussion

In this study, we found no significant associations between mean telomere length, as measured in blood leukocytes, and cancer status amongst *BRCA1* and *BRCA2* mutation carriers. i.e. we see no evidence that cancer cases from these families have differences in

mean telomere length compared to their unaffected, mutation-carrying relatives. This is in agreement with recent studies of telomere length and sporadic cancer risk in the general population (16,19-22). Unexpected, however, was the identification of a significant difference in mean telomere length between carriers and non-carriers of mutations in the *BRCA1* and *BRCA2* genes. In our study, mutation carriers (regardless of whether cancer-affected or unaffected) have longer telomeres than individuals from the same families without mutations. This was particularly apparent in families with *BRCA2* mutations (*P*-trend=0.0016). Expressed another way, *BRCA2* mutation carriers were 50% more likely to have a mean telomere length measurement in the longest quartile for length, compared with the shortest, than non-carriers; OR [Q1 (longest) vs. Q4 (shortest, referent)] = 1.50 (95%CI 1.25 – 1.77), *P*=0.001. This finding seems initially counter-intuitive, as the prevailing hypothesis has been that people at higher risk of developing cancer would have shorter telomeres than people at low risk.

Published literature does lend support to our findings. *BRCA1* or *BRCA2* knock-down or mutation is reported to variously increase *TERT* expression, increase telomerase activity and increase telomere length, but also to reduce the structural stability of the telomere and increase genomic rearrangement. Over-expression of BRCA1 has been shown to inhibit *TERT* expression and cause telomere shortening in human cancer cell lines (34,35). Conversely, others report that that decreased BRCA1 expression can regulate mean telomere length both by increasing telomerase expression and by increasing telomere length, even in cells lacking telomerase activity (36). In addition to binding the ‘shelterin’ complex of proteins, the telomere is also protected by its tertiary architecture and the T-loop formed using the G-rich single-stranded overhang at the 3’ telomere end. The length of this overhang, and thus the stability of the telomere, is regulated by BRCA1 and RAD50 such that over-expression of either protein increases T-loop length (36). BRCA1 expression knock-down by siRNA, in mammary epithelial cells *in vitro*, has also been shown to increase the frequency of chromosomal rearrangements, increase telomere attrition and lead to defective telomere capping (37-40). Similarly, it has been reported that breast tumours in *BRCA2* mutation carriers have significantly more numerous complex chromosomal changes compared with non-carriers, and chromosomal abnormalities characteristic of alternative lengthening of telomeres (ALT) activity have also been seen in $BRCA2^{-/+}$ cell lines (41-43). BRCA2 (together with RAD51) associates with the telomere during S phase of the cell cycle (44), and mutations in *BRCA2* (more so than *BRCA1*) can induce telomere fragility and shortening, suggesting an important role for BRCA2 in chromosome and telomere stability. BRCA2 is also reportedly important in the replication of the G-rich 3’ lagging strand and, consequently, in telomere length homeostasis (41). Based on these observations, it is not surprising that *BRCA1* and/or *BRCA2* carrier status has a pleiotropic effect on telomere length, independent of any association with cancer risk.

In our analysis of telomere length in cancer-affected versus unaffected *BRCA1* and *BRCA2* mutation carriers, there is little evidence of an association between mean telomere length and breast or ovarian cancer occurrence. Our findings do not support those of a smaller study, reported by Martinez-Delgado (28), in which telomere length was associated with ovarian cancer status, most significantly in women aged 41-50 years (*P*-trend= 4.9×10^{-47}).

One of the major advantages of the EMBRACE study design is that subjects were recruited as part of families that contained carriers, both affected and unaffected, and non-carriers. These samples have been treated identically from collection to storage, so there is less chance of these findings being due to artefacts in DNA processing. For the analysis of telomere length against disease risk, we utilised a weighted cohort approach. While the EMBRACE study is not a true cohort, the weighted cohort approach provides unbiased relative risk estimates while adjusting for the oversampling of affected carriers. A weakness of the current study is that cancer-affected individuals were sampled after diagnosis. It is therefore possible that the comparison of telomere length between cases and controls could be biased, if the measurement is affected by the diagnosis of the disease or treatment. This potential bias is similar to that in many case-control studies of telomere length. There may also be survival bias, if women with poor prognosis, and hence are less likely to be, have longer or shorter mean telomere length; however, studies to date have not shown consistent associations between telomere length and survival. A preferable study design would be to utlise samples from carriers taken before diagnosis, and evaluate the association with cancer risk prospectively. Unfortunately, the number of cancers diagnosed prospectively in cohorts of carriers, including EMBRACE, is currently too small to permit prospective analyses, but such analyses should be possible in the future. Notwithstanding, our results suggest that, if there is any association between telomere length and breast cancer risk in carriers, it is likely to be weak. As such, our results are consistent with the results from prospective studies in the general population, and not consistent with previous findings from retrospective case-control studies suggesting a strong association between telomere length and cancer risk. Thus, any previous consideration of telomere length as a potential biomarker for cancer risk seems misplaced (14).

It is possible that in *BRCA1* and *BRCA2* mutations carriers, longer telomere lengths (compared to their age-adjusted relatives) are maintained by derepression of telomerase but, evidently, maintaining telomere-length is insufficient to protect *BRCA* mutation carriers from cancer development. In a recent study, we found that SNPs in the *TERT* gene (encoding the major subunit of telomerase), which control mean telomere length, are largely independent of other *TERT* locus SNPs that alter risks of breast and ovarian cancer in the general population, as well as in *BRCA1* mutation carriers (45). The roles of TERT in maintaining telomere length and affecting cancer risk are largely separate. Evidence is thus mounting against the hypothesis that measures of mean telomere length (or genetic variants that control mean telomere length) could act as biomarkers for cancer risk.

In conclusion, our main and unexpected finding is that *BRCA1* and *BRCA2* mutation carriers have longer telomeres than their non-mutation carrier, non-cancer-affected relatives. These results suggest that telomere length is altered in *BRCA1* and *BRCA2* mutation carriers, but that this is not related to its effect on cancer risk. Our findings lend little support to the hypothesis that shorter mean telomere length predisposes to cancer, and indicate that mean telomere length measurements in blood DNA are unlikely to be useful biomarkers for cancer prediction.

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Table 1

Summary characteristics for the *BRCA1* and *BRCA2* carriers and non-carrier relatives used in the analysis. All individuals used for analysis were of self-reported white European ancestry. All individuals described below were included in the carrier status analysis shown in Table 2. Male participants and females on whom appropriate censoring data were not available were excluded from the weighted Cox regression analyses shown in Tables 3 and 4.

Characteristic		Non-carriers		*BRCA1* carriers		*BRCA2* carriers	
		Unaffected	Affected	Unaffected	Affected	Unaffected	Affected
Total number		**1636**	**52**	**797**	**831**	**791**	**715**
	Males♂	306	6	198	7	189	45
	Females♀	1330	46	599	824	602	670
Age at blood draw							
(mean, se)	All	45.9 (0.3)	55.4 (1.5)	42.8 (0.5)	50.5 (0.4)	44.2 (0.4)	53.9 (0.4)
	♀only	44.5 (0.3)	54.1 (1.5)	39.6 (0.4)	50.4 (0.4)	42.6 (0.5)	53.2 (0.4)
Age at censure							
(mean, se)	♀only	n/a	n/a	39.0 (0.5)	41.6 (0.4)	42.7 (0.6)	45.3 (0.4)
Relative telomere length							
(mean, se)	All	−10.5 (0.1)	−10.1 (0.6)	−10.3 (0.1)	−10.8 (0.1)	−9.7 (0.1)	−10.1 (0.2)
	♀only	−10.6 (0.1)	−10.0 (0.6)	−10.2 (0.2)	−10.8 (0.1)	−9.7 (0.2)	−10.1 (0.2)

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Table 2

Cancer status and quartile of mean telomere length in female *BRCA1* and *BRCA2* mutation carriers. Differences in telomere length (by quartile of length) between cancer-affected and unaffected mutation carriers are shown. Associations are presented as Hazard Ratios (HR) with 95% confidence intervals (95%CI). Analyses are adjusted for age, study plate, and relatedness. Weights were generated for this study based on time at risk before age at censoring, affected status and mutation type (*BRCA1* or *BRCA2*).

	Telomere length and cancer status HR (95%CI), *P*-het	
Relative telomere length	***BRCA1* mutation carriers 614 affected, 471 unaffected**	***BRCA2* mutation carriers 499 affected, 459 unaffected**
Q1 longest	1.00 ref	1.00 ref
Q2	0.91 (0.67 – 1.25), 0.57	1.26 (0.87 – 1.84), 0.23
Q3	1.27(0.71 –2.28), 0.42	1.89 (0.90 –3.98), 0.09
Q4 shortest	0.85 (0 37 – 1.98), 0.71	1.27 (0.49 –3.34), 0.62
Per quartile	0.96 (0.76 –1.22) *P*-trend = 0.76	1.17 (0.88 – 1.56) *P*-trend = 0.27

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Table 3

BRCA1 and *BRCA2* mutation carrier status and mean telomere length In all study individuals.

Differences in telomere length (δΔCt) between *BRCA* mutation carriers and non-carrier relatives in each study are shown. Associations are presented as β-coefficients with 95% confidence intervals (95%CI). Estimates are shown for all non-carriers compared to all carriers. Analyses are adjusted for age, study plate, relatedness and gender.

	Telomere length and carrier status β-coeff (95%CI)		
Relative telomere length	**All Carriers 3134 carriers, 1688 non-carriers**	***BRCA1* mutation carriers 1628 carriers, 1688 non-carriers**	***BRCA2* mutation carriers 1506 carriers, 1688 non-carriers**
All non-carriers	0.00 ref	0.00 ref	0.00 ref
All carriers	−0.056 (−0.091 to -0.021)	−0.038 (−0.079 to 0.003)	−0.067 (−0.108 to −0.026)
***P*-value**	0.0018	0.068	0.0016

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Table 4

BRCA1 and *BRCA2* mutation carrier status and mean telomere length in EMBRACE in all unaffected individuals.

Differences in telomere length (δΔCt) between *BRCA* mutation carriers and non-carrier relatives in each study are shown. Associations are presented as β-coefficients with 95% confidence intervals (95%CI). Estimates are shown for all unaffected non-carriers compared to unaffected carriers only. Analyses are adjusted for age, study plate, relatedness and gender.

	Telomere length and carrier status β-coeff (95%CI)		
Relative telomere length	**All unaffected carriers 1588 carriers, 1636 non-carriers**	**BRCA1 mutation carriers 797 carriers, 1636 non-carriers**	**BRCA2 mutation carriers 791 carriers, 1636 non-carriers**
Non-carriers	0.00 ref	0.00 ref	0.00 ref
Carriers	−0.056 (−0.098 to −0.013)	−0.041 (−0.094 to 0.011)	−0.069 (−0.123 to −0.016)
***P*-value**	0.011	0.12	0.011